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A gliotoxin model of occipital seizures in rats

Seyed M. Mirsattari [a,b,c,*], Bixia Shen [d], L. Stan Leung [a,d], Nagalingam Rajakumar [a,e]

[a] *Neuroscience Program in the Department of Graduate Studies, University of Western Ontario, London, Ontario, Canada*
[b] *Departments of Clinical Neurological Sciences, University of Western Ontario, London, Ontario, Canada*
[c] *Medical Biophysics, University of Western Ontario, London, Ontario, Canada*
[d] *Physiology & Pharmacology, University of Western Ontario, London, Ontario, Canada*
[e] *Anatomy & Cell Biology, University of Western Ontario, London, Ontario, Canada*

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Summary

Purpose: Intracortical microinjection of fluorocitrate, a reversible inhibitor of glial tricarboxylic acid (TCA), results in impaired glial metabolism and epileptic seizures. To determine the potential contribution of epileptic activities to the metabolic properties of fluorocitrate, we investigated the seizure-inducing property of fluorocitrate at different doses.
Methods: Twenty-seven male Sprague Dawley rats (250–400 g) were studied with chronically implanted electrodes and cannulae in the occipital cortices. A week after surgery, awake behaving rats were injected with 0.2 μl solution containing various concentrations of fluorocitrate or saline in the right occipital cortex; two sham-treated animals did not receive an injection. EEG was recorded with implanted electrodes. Thionin staining was used to verify injection sites. Twenty rats underwent immunohistochemistry for glial fibrilary acidic protein (GFAP) and neuronal nuclear-specific antigen (NeuN) 48 h after the injections.
Results: Seizures developed within an hour of injection in all the rats that received ≥0.8 nmol fluorocitrate and 2 of 4 rats that received 0.4 nmol fluorocitrate. Five of 12 animals that received ≥1.2 nmol fluorocitrate experienced status epilepticus. There was a significant increase in GFAP staining at the injection site in doses ≥0.8 nmol fluorocitrate. There was only mild neuronal loss revealed by NeuN staining at the injection site in the animals that had received 1.6 nmol flourocitrate.
Conclusion: This study shows that fluorocitrate results in focal epileptic seizures with secondary generalization in a dose-dependent manner, including low doses of this agent previously used for studies of brain metabolism.

* Corresponding author at: B10-108, London Health Sciences Centre, 339 Windermere Road, London, Ontario N6A 5A5, Canada. Tel.: +1 519 663 3448; fax: +1 519 663 3440.
E-mail address: smirsat2@uwo.ca (S.M. Mirsattari).

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Introduction

Epileptic seizures are evolving repetitive rhythmic discharges associated with behavioral changes[1–4] while interictal epileptic spikes (IEDs) are abnormal hypersynchronous electrical discharges of a population of neurons in a small region of the brain with no or minimal propagation so that they usually do not alter behavior.[5] Electroencephalogram (EEG) remains the primary tool in the study of epileptic activities. EEG tracing records the extracellular electrical field potentials generated by a population of cortical neurons, which amplifies small changes in the voltage difference between two inputs electrodes and displays them continuously with great temporal resolution.[6]

More than 30 percent of patients with epilepsy have inadequate control of seizures with drug therapy and are considered to have intractable epilepsy with recurrent seizures.[7] This underlies our limited understanding of the factors that trigger seizures in epileptic patients and requires greater attention to the potential role of the non-neuronal elements of the nervous system. Astroglial cells play a key role in energy metabolism and cycling between the inhibitory and excitatory neurotransmitters through glutamate-glutamine-gamma aminobutyric acid (GABA) shunt.[8] The participation of glial cells in seizure generation is not well known. Fluorocitrate is a reversible blocker of aconitase in tricarboxylic acid (TCA) cycle, which preferentially inhibits the glial TCA cycle and impairs glial metabolism. Fluorocitrate inhibits glial metabolism in *in vitro* preparations at doses ranging from 5–100 μM.[9] Intracerebral injection of fluorocitrate reduces glial glutamine,[10,11] but its effect has been demonstrated at a relatively larger volume of about 1 μl of 1 mM solution (i.e. 1 nmol).[12] Even larger intrathecal volume (i.e. 5 μl of 1 mM or 5 nmol) and similar systemic doses of fluorocitrate has been shown to induce seizures.[13] Direct spread of flourocitrate in such large volumes may result in loss of its cellular selectivity. In a previous study,[11] seizures resulted from a high concentration (6.4 mM in 0.125 μl or 0.84 nmol) of fluorocitrate injected intracortically into the frontal cortex in acute experiments. However, there are no published data on the epileptic properties of different doses of fluorocitrate despite its popularity to study cerebral metabolism using modern tools such as magnetic resonance spectroscopy (MRS).[9] The purpose of this study was to determine epileptic property of a fixed volume of fluorocitrate at various doses. Fluorocitrate was injected into the occipital cortex of SD rats in the present study.

Materials and methods

Animal preparations

This study was approved by the University of Western Ontario's Institutional Animal Care and Use Committee. Twenty-seven adult male Sprague Dawley (SD) rats (Charles River, QE) with body weight ranging from 250–400 g were housed in a 12 h dark and 12 h light cycle and had food and water *ad libitum*. The animals were anesthetized with sodium pentobarbital (65 mg/kg, intraparotenially (IP), MTC Pharmaceuticals, Cambridge, ON) and atropine methylnitrate (0.12–0.17 mg/kg, IP) was injected to prevent fluid accumulation in the

Table 1 Electrographic characteristics of the SD rats in this study

Drug	No. of animals per group	Average body weight (g)	Epileptic activity: duration and onset after injection
Sham-treated animals (no injection)	2	328.7 ± 22.	None
Saline control	3	294.7 ± 23.2	None
FLU 0.2 nmol mM	3	306 ± 12.5	None
FLU 0.4 nmol	4	323 ± 14.5	Recurrent seizures in 2/4 rats for over 30 min, onset at ~1 h
FLU 0.8 nmol	3	308 ± 5.7	Brief seizures in 3/3 rats for over 1 h, onset at ~45 min
FLU 1.2 nmol	5	302.8 ± 21.6	Recurrent seizures for over 2 h in 5/5 rats; onset <30 min; 2/5 rats with SE lasting ~3 h with onset within 1 min of injection
FLU 1.6 nmol	7	285.7 ± 27	Recurrent seizures for over 3 h in 7/7 rats; onset <15 min; 3/7 rats with SE lasting up to 6 h that began within 1 min of injection

FLU, fluorocitrate, and saline injected in a volume of 0.2 μl; SE, status epilepticus.

airway. They were then placed in a stereotaxic apparatus (David Kopf Instruments, Inc., Tujunga, CA) to implant 6 EEG electrodes (125 μm diameter stainless-steel wires electrodes coated with Teflon except at the tips) and two 27 gauge stainless-steel canuulae for guiding subsequent infusion by a 33 gauge inner canuula. The animal head was shaved and scalp incised in order to expose the skull. The homologous regions of the frontal lobes (B +1.0, L ±2.0, D 1.6), hippocampus (B −3.5, L ±2.5, D 3.3), and occipital lobes (B −6.0, L ±4.0, D 1.6) were implanted with the electrodes, which were secured in place with dental cement. Some animals had additional two electrodes in the parietal (B −0.8, L ±4.2, D 1.6) regions. The canuulae were inserted in the same burr holes as the occipital electrodes. The coordinates were obtained from a stereotaxic rat brain atlas.[14] The animals were allowed to recover from the surgery for at least 1 week. Fluorocitrate solutions were prepared according to a published method[15] from DL-fluorocitric acid barium salt (F9634, $C_{12}H_8F_2O_{14}Ba_3$, M.W. 826.16, purity 95%, Sigma–Aldrich Canada Ltd., Oakville, ON). There were ≥3 animals in each group, plus 2 sham-treated rats that underwent the same surgery but they did not receive any injections (Table 1). On the day of experiment, 0.2 μl of saline or 0.2, 0.4, 0.8, 1.2, and 1.6 nmol of fluorocitrate was injected through the right occipital canuula 1.6 mm below the skull surface using a polyethylene 50 tube attached to a Hamilton microlitre syringe (72–1742, 0.5 μl volume with a 32 gauge needle, Harvard Apparatus, Inc., Saint Laurent, QE). The canuulae in the left occipital lobe were used in two cases that the right-sided canuulae were blocked. EEG was recorded by a Grass Instruments Model 8–10 polygraph or a digital EEG (XLTEK, Oakville, ON) using a laptop computer. EEG was monitored for maximum of 6 h (i.e. till the termination of epileptic discharges) after each injection and again half an hour per day until their sacrifice 48 h after the injections. They were then given a bolus of 45% urethane 1.3 g/kg IP (Sigma–Aldrich Inc., St. Louis, MO) and perfused transcardially with 100 ml of 0.9% saline followed by 500 ml of fixative consisting of 4% freshly depolymerized paraformaldehyde (BDH Inc., Toronto, ON) in 0.1 M phosphate buffer (PB, pH 7.4) using a perfusion pump (Masterflex C/liter™ pump

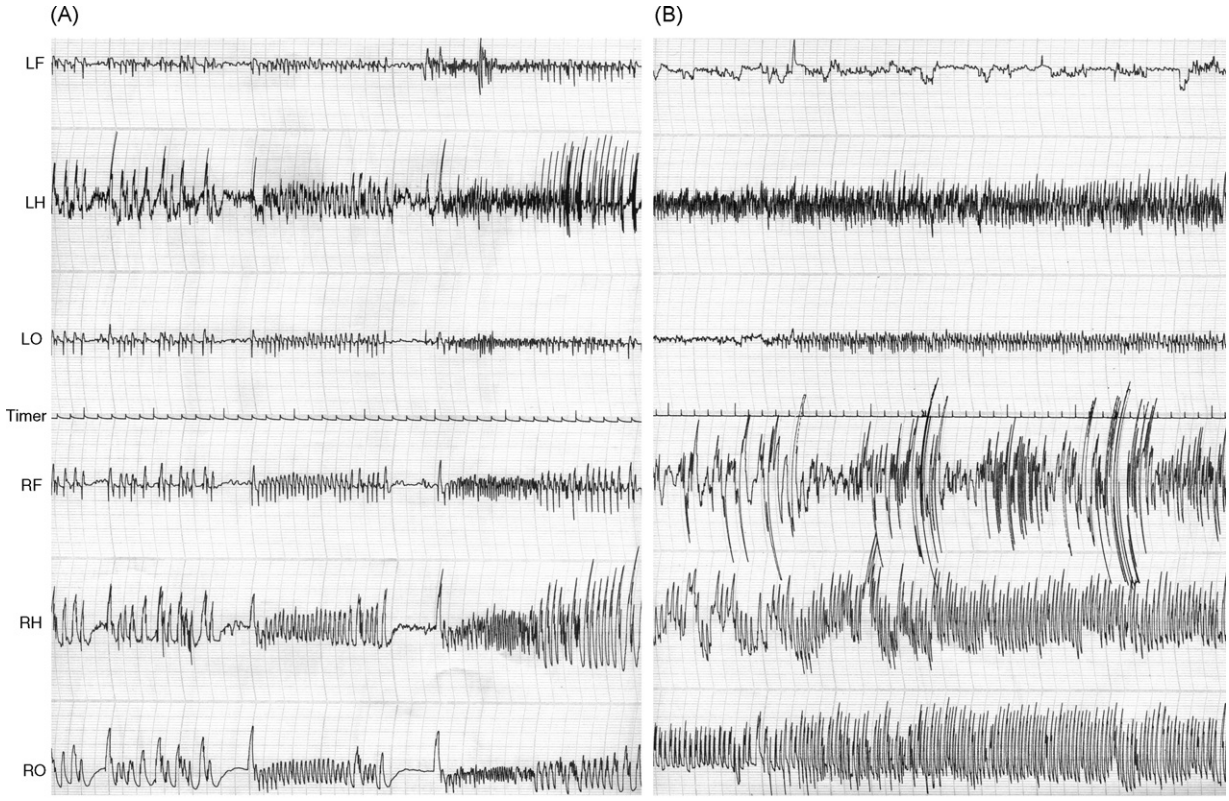

Figure 1 Fluorocitrate induced seizures in an undrugged and awake SD rat (240 g) given 0.2 μl of 8 mM (1.6 nmol) of fluorocitrate in the right occipital cortex (RO). Note extensive spread to the left occipital cortex (LO) and the right hippocampus (RH) as well as prominent spread to the left hippocampus (LH), left and right frontal lobes (F). (A) and (B) are both segments of the same seizure. Referential intracranial recordings with the reference in the skull and recordings done on a polygraph manufactured by Grass Co. Each small square represents 1 s in time domain. Sensitivity = 1 mV/Cm.

system, Cole—Parmer Instrument Co., Vernon Hills, IL) via the left ventricle of the heart for histologic confirmation of the injection site using thionin staining and subsequent immunocytochemical examination.

Immunohistochemistry

Brain of each animal was removed and cryoprotected in 18% buffered-sucrose for subsequent histological studies. Coronal sections (40 μm) through the entire brain were cut on a freezing microtome, collected in PB, and kept in storage solution containing 30% glycerol, 30% ethylene glycol (Sigma—Aldrich Inc., St. Louis, MO) in 0.1 M PB at −20 °C. The injection site and location of the recording electrodes were verified under the light microscopy after staining with thionin in all the animals. Three animals per each dose of fluorocitrate and saline and the two sham control animals (i.e. $n = 20$) were studied with glial fibrilary acidic protein (GFAP) and a neuronal marker (NeuN) immunostaining. Briefly, free floating brain sections were removed from storage solution, washed in 0.1 M PB and then placed into blocking serum consisting of 10% non-immune normal goat serum (Sigma—Aldrich Inc., St. Louis, MO) and 0.1% Triton X-100 (Sigma—Aldrich Inc., St. Louis, MO) in PB for 1 h at room temperature. After blocking nonspecific bindings, the sections were incubated in GFAP antibody (rabbit polyclonal, 1:500, Dakocytomation, Denmark) or NeuN (mouse monoclonal, 1:20,000, Abcam Inc., Cambridge, MA) in PB containing 1% bovine serum albumin (BSA, Sigma—Aldrich Inc., St. Louis, MO) for 48 h at 4 °C. The sections were washed in PB and then incubated in biotinylated goat anti-mouse antibody at 1:200 (Bio/Can Scientific, Mississagua, ON) in PB containing 1% BSA for 1 h at room temperature. The sections were washed in PB and then incubated in avidin—biotin horseradish peroxidase complex (ABC Elite; Vector Laboratories, Burlingham, CA) for 1 h at room temperature. After washing in PB, they were incubated in the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma—Aldrich Inc., St. Louis, MO) and then hydrogen peroxide (0.003%) was added to initiate the peroxidase reaction. Sections were washed, mounted on chrome-alum-coated slides, and allowed to air dry. They were then dehydrated in a series of ascending alcohols (70%, 95%, 100% twice for

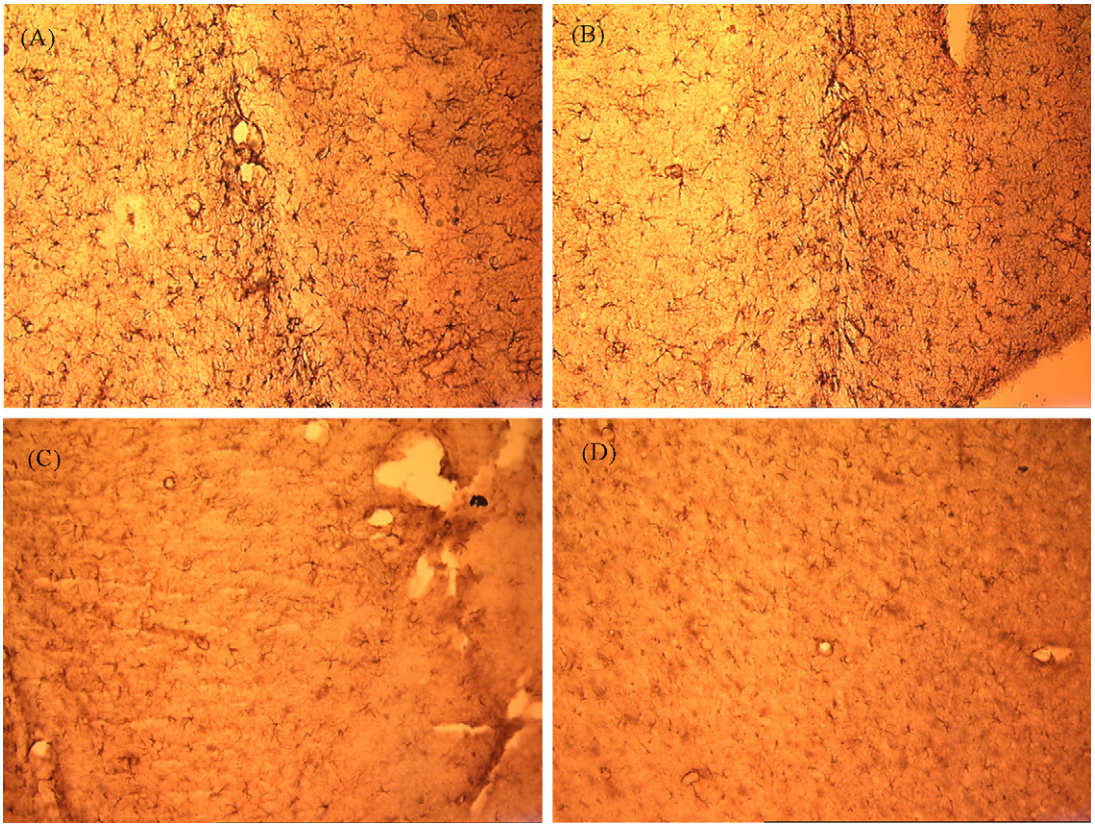

Figure 2 GFAP staining in the same rat shown in Fig. 1 (A and B) 48 h after the injection of fluorocitrate and a control animal (C and D). (A) and (C): sites of injection, (B) and (D): contralateral sites. Note the reactive astrocytosis at the injection site (A) only and not in the contralateral site (B). Magnification 16×.

2 min each), cleared in xylene for 5 min twice and coverslipped with Entalan mounting medium (BDH Inc.). To control for specificity of immunostaining, some sections were processed as above but without the primary or secondary antibody. The immunohistochemistry method for NeuN followed previously published.[16] Immunohistochemical labeling was examined using an Axioskop miscroscope (Zeise) under transmitted light, images were photographed and number of NeuN-labeled neurons were quantified in 200 μm width axial strip of the occipital cortex using Image J Image Analysis Software (NIH), while GFAP-labeled cells in this area were quantified by direct visual counting. Quantitative data were analyzed using one-way ANOVA followed by Turkey's post hoc test with alpha set at 0.05.

Results

All 15 rats injected with ≥0.8 nmol of fluorocitrate and 2 of the 4 rats with 0.4 nmol flourocitrate developed seizures within an hour of the injection, but none of the 3 rats injected 0.2 nmol of fluorocitrate experienced seizures (Table 1 and Fig. 1). Two of 5 rats that received 1.2 nmol of fluorocitrate and 3 of 7 rats that received 1.6 nmol of fluorocitrate developed status epilepticus (SE) with violent motor seizures lasting greater than 1 h and required diazepam 5 mg/kg IP to terminate the seizures. These rats developed seizures within 1 min of injecting fluorocitrate. The average delay to the onset of the seizures was 30 min. None of the rats had seizures during the subsequent recording days, except for the recurrence of a single motor seizure in one rat 24 h after receiving 0.8 nmol of fluorocitrate. There was a dose-dependent increase in the intensity of the seizures and they frequently become secondarily generalized. During the seizures, the animals either had motionless stare or violent jumps and running in circles in the cage. There were very few IEDs and they were seen with fluorocitrate doses of ≥0.8 nmol and only within the first 6 h of the recording. Sham-treated animals and those receiving saline injections did not have any epileptic discharges at any time of the recording.

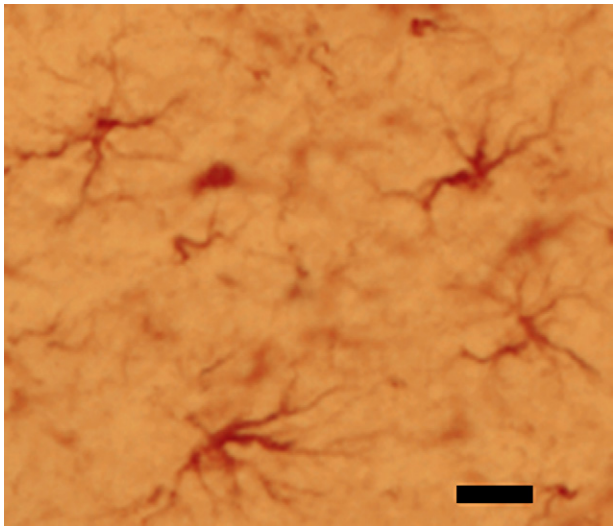

Figure 3 Higher magnification image showing GFAP-labeled cells in the occipital cortex of 0.8 nmol fluorocitrate-injected rat (bar: 25 μm).

The injection sites were verified by thionin staining in all the animals. There was a dose-dependent significant increase in GFAP staining at the injection site with enlarged astrocytes that had swollen processes in all the study animals that received ≥0.8 nmol of fluorocitrate (Figs. 2 and 3). Only minimal GFAP staining at the injection site was seen with 0.4 nmol of fluorocitrate but none with 0.2 nmol of fluorocitrate, saline injection, or sham-treated animals (Table 2). NeuN staining demonstrated minimal neuronal loss at the injection site only with the highest dose of fluorocitrate (i.e. 1.6 nmol) (Fig. 4). Otherwise, there was no neuronal loss (Table 2).

Discussion

Fluorocitrate induced a dose-dependent epileptic activity in SD rats. At low doses (0.4—0.8 nmol),

Table 2 Changes in neuronal and glial numbers quantified in a 200 μm width radial segment of the full thickness of the occipital cortex adjacent to the injection site

Drug	GFAP immunoreactive cells	NeuN immunoreactive cells
Saline control	4.8 ± 2.7	2864.4 ± 186.8
Fluorocitrate 0.2 nmol	5.3 ± 4.8	2917.6 ± 369.6
Fluorocitrate 0.4 nmol	8.1 ± 6.2	2783.5 ± 258.9
Fluorocitrate 0.8 nmol	31.4 ± 8.1*	2804.7 ± 164.8
Fluorocitrate 1.2 nmol	35.7 ± 5.9*	2668.6 ± 203.7
Fluorocitrate 1.6 nmol	51.8 ± 8.6*	2690.4 ± 348.8

All values were indicated as the means ± standard error of the number of immunoreactive cells in the occipital cortex adjacent to the injection site. *$p < 0.05$ compared with control group.

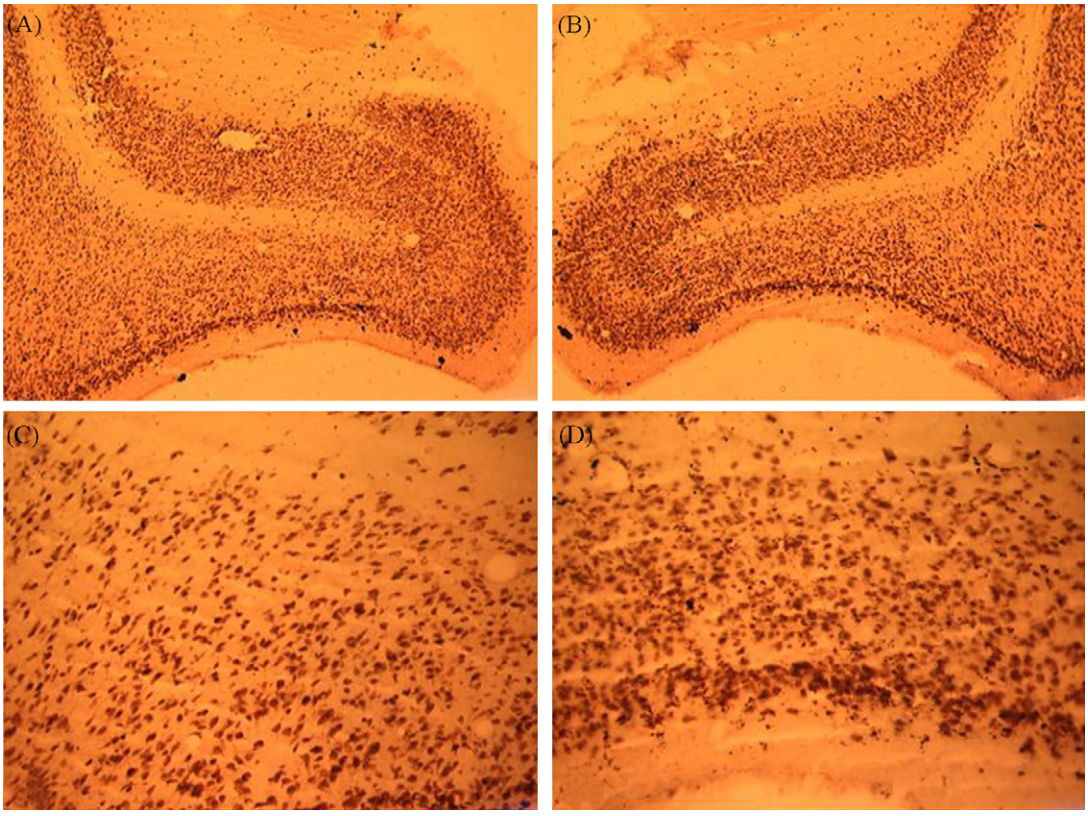

Figure 4 NeuN immunostaining in the same rat as in Figs. 1 and 2 48 h after the injection of fluorocitrate. (A) Injection site and (B) contralateral site. (C) and (D) are higher power views of A and B, respectively. Note mild neuronal loss at the injection site. Magnification 16×.

focal seizures with secondary generalization resulted. At high doses (1.2—1.6 nmol), SE was found in about half the injected animals. Epileptic spikes were exceedingly infrequent and seizures were either associated with motionless stares or violent motor signs (jumping and running in circles). This study confirms that glial cells are important in generating epileptic discharges. Therefore, the diverse nature of human epilepsy[17] and animal models may be due to varying contributions of the glia cells and neurons.

Willoughby et al.,[11] originally reported that epileptic discharges in the frontal cortex were induced with local injection of 0.84 nmol of fluorocitrate, resulting in motor convulsions in 4 of 7 injected rats. However, Willoughby et al.,[11] injected fluorocitrate under 2% halothane anesthesia, and while the EEG and behavior were observed after the cessation of halothane, the surgical procedure and lingering halothane could have affected the EEG and behavioral responses. By using chronically implanting electrodes, the present study established that a lower dose of fluorocitrate induced epileptic discharges and a higher dose induced SE.

The exact mechanisms of glial-mediated seizures remain to be determined.[18] However, extracellular glutamate and potassium (K^+), which are normally cleared by glial cells, are likely important contributors.[19—21] Fluorocitrate disrupts glial function and allows local accumulation of glutamate and K^+, partly derived from normal activity, such that epileptiform activity may arise. Synaptic transmission, gap junctions, ephaptic transmission, antidoromic spread, and ionic field potentials[22—26] may all play a role in seizure spread induced by fluorocitrate, similar to seizures induced by other agents. Future studies that employ combination of fluorocitrate and other agents will be valuable in understanding the interaction of neural and glial elements in generating, maintaining, and propagating epileptic discharges.

The high incidence of secondarily generalized seizures and SE in fluorocitrate-induced seizures demonstrates a potentially important role for the glial cells in the rapidly propagating seizures, SE, and medically refractory seizures. There was a relative paucity of epileptic spikes in the animals injected with fluorocitrate, which can occur in some epileptic patients.[17] Low doses of fluorocitrate that do not cause seizure can be used to study early metabolic changes that may lower seizure threshold. The epileptic properties of flourocitrate

demonstrated in this study could have implications for interpretation of various metabolic studies using fluorocitrate including those involving MRS. Finally, the use of flourocitrate to study brain metabolism should be limited to its non-epileptic doses demonstrated in this study.

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